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ABSTRACT

Studies suggest a correlation between ethanol consumption and periodontal disease. We hypothesized that elevated levels of blood reactive oxygen species following ethanol consumption may increase inflammation in periodontal tissue. Rats were divided into 4 groups (6-7 rats/group). Two groups were fed an ethanol-containing liquid diet, and 2 groups were fed a pair-fed control diet. In one of each dietary group, periodontitis was ligature-induced, while the other group was left unligated. Chronic ethanol feeding alone decreased the ratio of reduced/oxidized glutathione and increased 8-hydroxydeoxyguanosine and tumor necrosis factor (TNF)- α levels in the gingiva. Blood hydroperoxides were also increased. In ligature-induced periodontitis lesions, ethanol feeding enhanced polymorphonuclear leukocyte infiltration and TNF- α expression. The results suggest that chronic alcohol consumption increased periodontal inflammation, oxidative damage, and TNF- α production and had an additive effect on polymorphonuclear leukocyte infiltration and gingival oxidative damage, increasing the severity of periodontal inflammation in the ligature model.

KEY WORDS: ethanol, periodontitis, oxidative stress, animal studies.

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INTRODUCTION

Alcohol-related disorders are among the challenging health problems with far-reaching medical, social, and economic consequences. Recent studies have indicated an epidemiological association between alcohol consumption and periodontal disease. A cross-sectional study of 13,198 individuals in the Third National Health and Nutrition Examination Survey (NHANES III) suggested that alcohol consumption was associated with increased severity of clinical attachment loss in a dose-dependent fashion (Tezal *et al.*, 2004). A longitudinal study including 39,461 male health professionals, aged 40 to 75 yrs, also demonstrated that alcohol consumption may be an independent modifiable risk factor for periodontal disease (Pitiphat *et al.*, 2003).

Chronic alcohol (ethanol) consumption leads to the generation of excess amounts of reactive oxygen species (ROS) in the liver (Das and Vasudevan, 2007), and such conditions increase the blood levels of ROS (Barden *et al.*, 2007; Kapaki *et al.*, 2007). Since tissue oxidative damage, induced by ROS, is involved in the pathogenesis of periodontal disease (Chapple and Matthews, 2007), increased circulating ROS following ethanol consumption may be detrimental to periodontal health.

Several studies have provided possible biological explanations for the detrimental effects of ethanol consumption on periodontitis risk. Ethanol impairs neutrophil function (Szabo, 1999) and bone metabolism (Trevisiol *et al.*, 2007), which may lead to progression of periodontitis. High ethanol consumption stimulated monocyte production of TNF- α and interleukin-6 in the gingival crevice (Offenbacher, 1996). However, it is unclear how chronic ethanol consumption affects histological changes in the periodontium and gingival oxidative stress.

We hypothesized that ethanol consumption might increase ROS, not only in the blood, but also in the gingiva, and might induce destruction of the periodontium. The purpose of this study was to examine the effect of chronic ethanol feeding on the periodontium and gingival oxidative damage with and without ligature-induced inflammation in a rat model.

MATERIALS & METHODS

Experimental Design

All experimental procedures were performed in accordance with regulations of the Animal Research Control Committee of Okayama University Dental School. Twenty-six male Wistar rats (10 wks old) were randomly divided into 4 groups. The first 2 groups were provided an *ad libitum* liquid diet containing ethanol at 36% of total caloric value (Lieber-DeCarli liquid diet; Oriental Yeast Co., Tokyo, Japan) for 8 wks (Lieber *et al.*, 2004), and either received no treatment (Ethanol group, $n = 6$) or had ligature-induced periodontitis (Combination group, $n = 7$) for 4 wks prior to the end of the experimental period. The remaining 2 groups were pair-fed with the same diet, except that ethanol was

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isocalorically replaced with carbohydrate for 8 wks, and either received no treatment (Control group, $n = 6$) or had ligature-induced periodontitis (Periodontitis group, $n = 7$) for 4 wks prior to the end of the experimental period. Animals were not provided with additional *ad libitum* water. A cotton ligature was placed in a subgingival position of the mandibular first molars.

Histological Analysis

The animals were killed after the experimental period, under general anesthesia. Samples of the left mandibular molar regions were resected from each rat and immediately fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 1 day. Mandibular samples were further decalcified with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 2 wks at 4°C. Formalin-fixed tissue samples were embedded in paraffin and stained with hematoxylin and eosin.

Immunohistochemical staining of TNF- α was performed with a Histofine Simple Stain MAX PO (G) kit (Nichirei Co., Tokyo, Japan). Briefly, deparaffinized tissue sections were immersed in methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The sections were treated consecutively at 4°C with an anti-TNF- α antibody (R&D Systems, Minneapolis, MN, USA) (diluted 1:200) overnight, and with a secondary antibody (Fab') with peroxidase complex for 30 min. Peroxidase staining was performed for 5-10 min with a solution of 3,3'-diaminobenzidine tetrahydrochloride in 50 mmol/L Tris-HCl (pH 7.5) containing 0.001% hydrogen peroxide. The sections were counterstained with Mayer's hematoxylin. We established the specificity of staining by showing isotype antibody controls and by confirming prevention of positive staining when the antibody was previously treated with standard TNF- α (Biosource International, Camarillo, CA, USA) in the immunohistochemical procedure.

A single examiner, blind to the treatment assignment, performed the following histometric analyses using a light microscope. Gingival sections stained with hematoxylin and eosin were used for evaluation of the degree of apical migration of the junctional epithelium, alveolar bone resorption, and polymorphonuclear leukocyte infiltration. The distances between the cemento-enamel junction and the alveolar bone crest and between the cemento-enamel junction and the most apical portion of the junctional epithelium were measured with a graticule at a magnification of $\times 200$. The polymorphonuclear leukocytes of the connective tissue subjacent to the junctional epithelium in 2 standard areas [0.05 mm (depth) \times 0.1 mm each] were counted under a magnification of $\times 400$ (Ekuni *et al.*, 2003). The numbers of TNF- α -positive fibroblasts and total fibroblasts in standard areas (0.1 mm \times 0.1 mm each) adjacent to the cementum within the periodontal ligament (5 serial areas from the top of the periodontal ligament) were determined (Tomofuji *et al.*, 2005), and the ratios of TNF- α -positive fibroblasts to total fibroblasts were calculated. Three sections were selected from each rat, and means of histological data were calculated for each rat. We evaluated intra-examiner reproducibility by double-scoring 15 randomly selected sections at two-week intervals. Agreement within one cell of total fibroblasts and TNF- α -positive fibroblasts was 93.3% and 86.7%, respectively.

Determination of Glutathione and 8-OHdG Levels in Gingival Tissue

Gingival biopsy samples of the right mandibular molar regions were used for evaluation of the anti-oxidative status and oxidative damage. All measurements were performed in triplicate. The

sample was homogenized by the BioMasher single-use homogenizer (Omni International, Marietta, GA, USA). Using the supernatant of the homogenates, we determined the total glutathione [reduced-form glutathione (GSH) + oxidized-form glutathione (GSSG) level] and GSSG levels with a colorimetric microplate assay kit (Dojindo Laboratories, Tokyo, Japan) (Vandeputte *et al.*, 1994). GSSG was determined after GSH was blocked with 2-vinylpyridine. The GSH/GSSG ratio in each sample was calculated. The assay had a sensitivity of 1 $\mu\text{mol/L}$.

From the precipitate of gingival homogenates, mitochondrial DNA was isolated by means of a DNA extraction kit (Wako, Osaka, Japan), and the concentration of 8-hydroxydeoxyguanosine (8-OHdG) in the isolated mitochondrial DNA was analyzed with the use of an enzyme-linked immunosorbent assay kit (Japan Institute for the Control of Aging, Shizuoka, Japan) (Sanbe *et al.*, 2007). The kit can measure 8-OHdG values ranging from 0.125 to 10 ng/mL.

Measurement of Blood Hydroperoxide Concentration

At 8 wks, blood samples were drawn from the heart by means of a heparinized syringe, and plasma was obtained by centrifugation in a plasma separator tube (Sekisui Chemical Co., Tokyo, Japan). As an index of blood ROS, the hydroperoxide concentration was determined by means of a free radical elective evaluator (Diacron, Grosseto, Italy) (Papageorgiou *et al.*, 2005). This analysis was based on the ability of transition metals to catalyze, in the presence of peroxides, the formation of free radicals, which were trapped by an alchiamine. In brief, 20 μL plasma and 1.2 mL buffered solution (R_2 reagent) were mixed in a cuvette, and a 20- μL quantity of chromogen substrate (R_1 reagent, the aromatic amine *N,N*-diethylparaphenyldiamine) was added to the cuvette. After being shaken to mix, the cuvette was incubated for 5 min at 37°C. The absorbance increase at 505 nm was monitored for 5 min. The results of this method are expressed in conventional units (CARR units): 1 CARR unit corresponds to 0.8 mg/L H_2O_2 .

Statistical Analysis

The Mann-Whitney *U* test was used for comparisons between the Control and Ethanol groups, or between the Periodontitis and Combination groups, with a statistical software package (SPSS 13.0J for Windows; SPSS Japan, Tokyo, Japan). Sample size was calculated by the nQuery Advisor (Statistical Solutions, Saugus, MA, USA), based on the results of our previous study (Tomofuji *et al.*, 2005). A sample size of 6 *per* group was required for detection of a significant difference (80% power, two-sided 5% significant level).

RESULTS

No significant differences in weight gain or food consumption were detected among the 4 groups.

The distance between the cemento-enamel junction and the alveolar bone crest, the distance between the cemento-enamel junction and the most apical portion of the junctional epithelium, and the density of polymorphonuclear leukocytes were greater in the Ethanol group than in the Control group (Fig. 1). Although the density of polymorphonuclear leukocytes was higher in the Combination group compared with the Periodontitis group, ethanol did not affect ligature-induced bone resorption or apical migration of the junctional epithelium.

TNF- α was strongly detected in the cytoplasm of

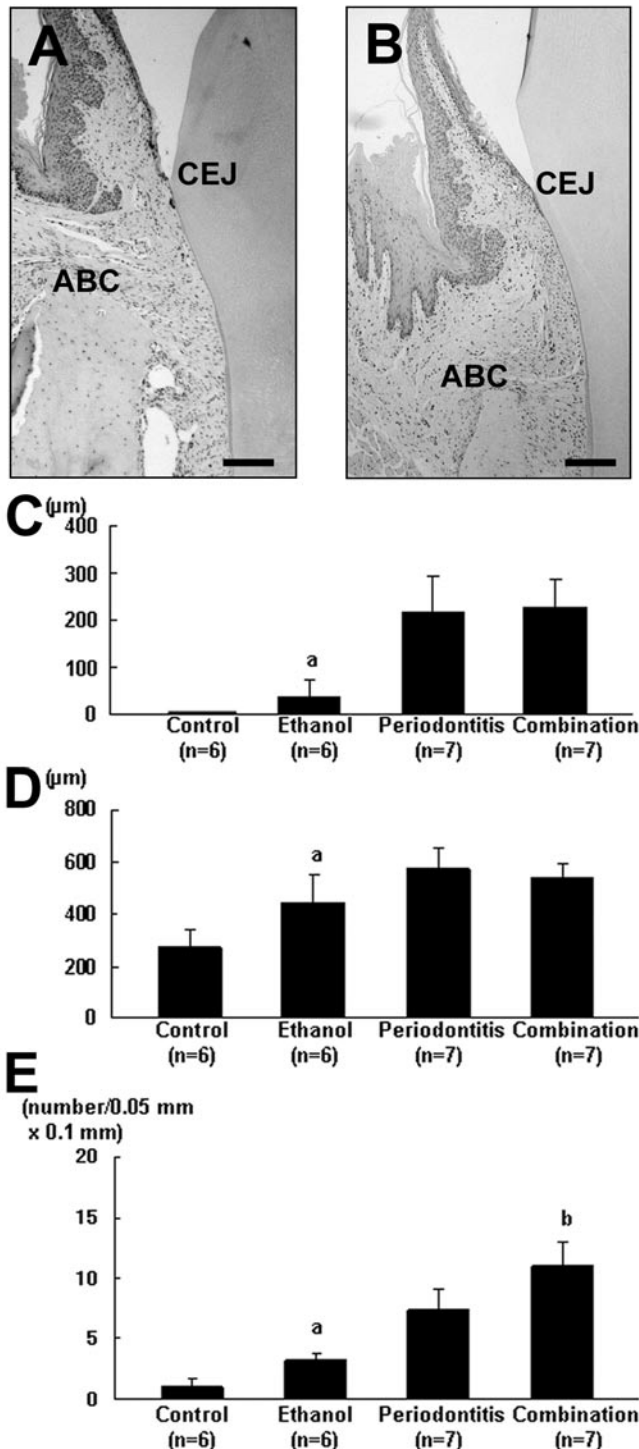


Figure 1. Pathological changes in rat periodontal tissue. While the Control group showed no pathological changes (A), the Ethanol group showed moderate alveolar bone resorption (B). Measures of linear distances between the cemento-enamel junction and apical portion of the junctional epithelium (C), linear distances between the cemento-enamel junction and alveolar bone crest (D), and polymorphonuclear leukocyte density (E) for the 4 experimental groups (mean ± SD). ABC, alveolar bone crest; CEJ, cemento-enamel junction. Bar scale = 50 μm. ^ap < 0.05, compared with the Control group. ^bp < 0.05, compared with the Periodontitis group.

periodontal ligament fibroblasts (Fig. 2). The ratio of TNF-α-positive fibroblasts to the total fibroblasts was 3.7 times higher

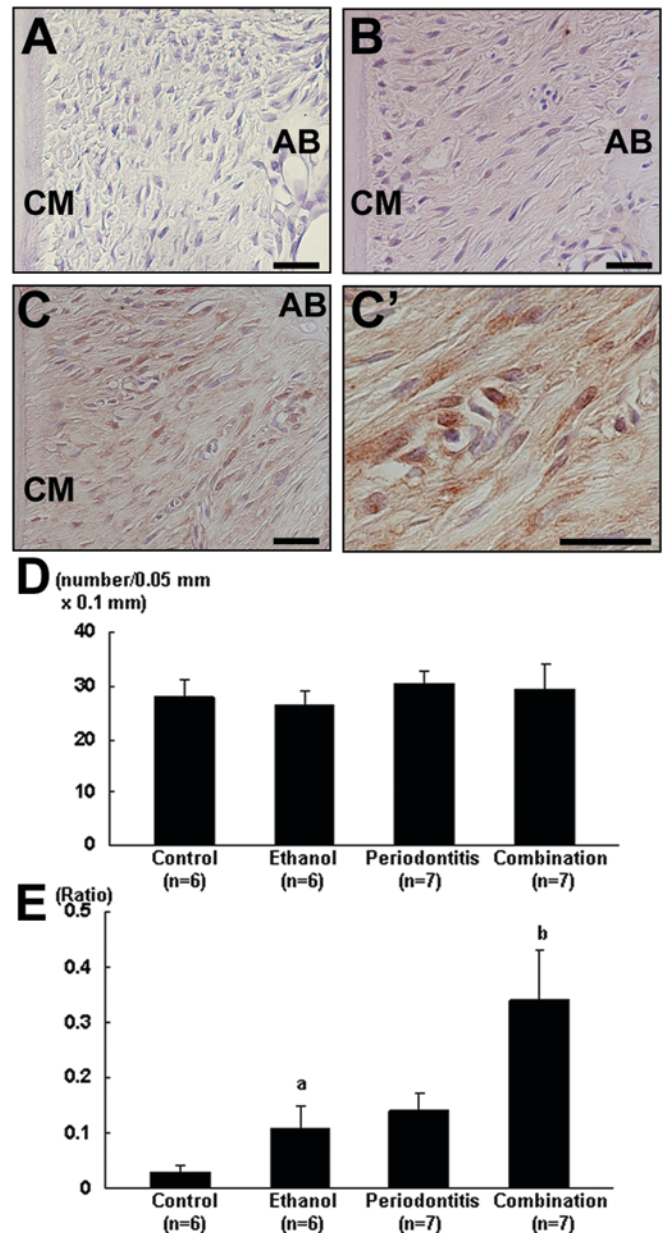


Figure 2. TNF-α-positive periodontal ligament fibroblasts in rats. Staining with isotype control immunoglobulin showing absence of reactivity (A). Few TNF-α-positive fibroblasts with brown-stained cytoplasm were found in the Control group (B). More TNF-α-positive fibroblasts were observed in the Combination group (C,C'). Fibroblast counts (D) and ratios of TNF-α-positive fibroblasts to total fibroblasts (E) for the 4 experimental groups (mean ± SD). AB, alveolar bone; CM, cementum. Scale bar = 25 μm. ^ap < 0.05, compared with the Control group. ^bp < 0.05, compared with the Periodontitis group.

in the Ethanol group than in the Control group, and 2.4 times higher in the Combination group than in the Periodontitis group.

Gingival levels of 8-OHdG and the GSH/GSSG ratios in the Ethanol group showed a significant increase of 79% and a significant decrease of 38%, respectively, compared with the Control group (Table 1). Gingival levels of 8-OHdG and the GSH/GSSG ratios in the Combination group also showed a

Table 1. The Levels of Gingival Anti-oxidant and Oxidative Damage in Rats

	Control (n = 6)	Ethanol (n = 6)	Periodontitis (n = 7)	Combination (n = 7)
GSH (mmol/L/g)	2.39 ± 0.37	1.82 ± 0.29 ^a	1.64 ± 0.24	1.49 ± 0.23
GSH/GSSG ratio	25.4 ± 4.5	15.6 ± 4.3 ^a	21.1 ± 2.0	8.5 ± 2.1 ^b
8-OHdG (ng/mg mtDNA)	0.42 ± 0.07	0.74 ± 0.10 ^a	0.90 ± 0.13	1.27 ± 0.22 ^b

Values are presented as means of 6-7 rats ± SD.

^a p < 0.05, compared with the Control group, Mann-Whitney U test.

^b p < 0.05, compared with the Periodontitis group, Mann-Whitney U test.

Table 2. The Levels of Plasma Hydroperoxide in Rats (CARR U)

Control (n = 6)	Ethanol (n = 6)	Periodontitis (n = 7)	Combination (n = 7)
242 ± 17	368 ± 46 ^a	335 ± 26	371 ± 35

Values are presented as means of 6-7 rats ± SD.

^a p < 0.05, compared with the Control group, Mann-Whitney U test.

significant increase of 72% and a significant decrease of 60%, respectively, compared with the Periodontitis group.

The blood hydroperoxide level in the Ethanol group was higher than that in the Control group and similar to that found in the Periodontitis group (Table 2); however, no significant differences were found between the Periodontitis and Combination groups.

DISCUSSION

Ethanol consumption increased blood levels of hydroperoxide and induced polymorphonuclear leukocyte infiltration in gingiva, while reducing the GSH/GSSG ratio within the gingiva. In addition, the distance between the cemento-enamel junction and the alveolar bone crest in rats without induction of periodontitis was increased by ethanol feeding, which indicates alveolar bone resorption. These results suggest that ethanol consumption can cause destruction of the periodontium and is associated with local alterations in redox balance.

In the periodontitis-induced rats, ethanol consumption enhanced periodontitis-induced oxidative damage of gingival tissues and polymorphonuclear leukocyte infiltration. Furthermore, the effects of ethanol consumption and induction of periodontitis on expression of TNF- α in fibroblasts were additive. The results indicate that ethanol consumption could increase oxidative damage of gingival tissues and promote the progression of periodontitis.

Several mechanisms might be involved in the periodontal destruction induced by ethanol consumption. Oxidative stress may cause direct damage to cells and tissues, as well as stimulating production of pro-inflammatory cytokines (Lee and Burckart, 1998). In this study, ethanol consumption for 8 wks resulted in a 79% increase in 8-OHdG formation and a 38% decrease in the GSH/GSSG ratio. Furthermore, we observed that the number of TNF- α -positive periodontal ligament fibroblasts was increased by ethanol consumption. TNF- α directly induces bone resorption in the periodontal tissue (Graves and Cochran, 2003). Increased gingival oxidative stress following ethanol consumption would lead to progression of periodontitis with the production of TNF- α . However, because TNF- α expression was strongly found in the periodontal ligament, it is also possible that it was induced where ROS or oxidative damage was not present.

Periodontitis increases tissue oxidative stress by potentiating inflammatory responses (Chapple, 1997; Chapple and Matthews, 2007). In our study, ligature-induced periodontitis increased the gingival 8-OHdG level. This observation is in

agreement with previously reported findings showing that lipid peroxidation increased in a ligature-induced periodontitis rat model (Di Paola *et al.*, 2004). Furthermore, the combination of ethanol consumption and ligature-induced periodontitis decreased the GSH/GSSG ratio and increased the 8-OHdG level greater than in only ligature-induced periodontitis, suggesting that ethanol consumption and ligature-induced periodontitis have an additive effect on gingival oxidative damage.

The influence of ethanol consumption on the periodontal tissue was different when periodontitis was induced: Only polymorphonuclear leukocyte infiltration increased due to ethanol consumption in ligature-induced periodontal lesions, while ethanol feeding alone caused apical migration of the junctional epithelium, alveolar bone resorption, and polymorphonuclear leukocyte infiltration. The degree of alcohol-induced effects differed according to the cell type (Schleifer *et al.*, 2006), and our results indicated that polymorphonuclear leukocytes were highly susceptible to ethanol consumption. Both oxidative damage and polymorphonuclear leukocyte infiltration have been observed in alcohol-induced lung damage in rats (Aytacoglu *et al.*, 2006), findings in agreement with those of the present study.

The ethanol-containing liquid diet is commonly used to study the medical consequences of ethanol consumption in animal models (Lieber *et al.*, 2004). We used a high dose of ethanol at 36% of total calories in the diet, because, in rats, it is known to produce high blood levels of ethanol (26-43 mM) and certain characteristics of abnormal liver metabolism that precede the onset of human alcoholic liver disease (Hunter *et al.*, 1975; Donohue *et al.*, 1987).

This study used a ligature-induced periodontitis model, because such a periodontal lesion mimics several features of human periodontitis, including the inflammatory cellular infiltrate, loss of attachment, and alveolar bone resorption (Di Paola *et al.*, 2004; Seto *et al.*, 2007). In addition to the traditional parameters of periodontitis, we further examined the gingival anti-oxidant level and oxidative damage in the ligature-induced inflammation model. However, it would be more reliable to use a ligature along with the application of bacterial pathogens (*e.g.*, *Porphyromonas gingivalis*) as a rat model that relates to human periodontitis (Liu *et al.*, 2006; Li and Amar, 2007). Furthermore, ligature-induced periodontal inflammation is an acute model of periodontitis, and not directly equivalent to the chronic disease in humans.

Our study had other limitations. We performed only histological analyses to obtain the data for TNF- α . Further

detailed investigations, such as real-time PCR, for the assessment of TNF- α mRNA levels might be needed to improve the reliability of our data. In addition, because ethanol consumption results in hepatic inflammation (Jaruga *et al.*, 2004), increased circulating inflammatory molecules, such as C-reactive protein, may also affect the periodontal health. Determination of blood C-reactive protein levels will be required to elucidate the mechanism of action of ethanol in the present model.

In conclusion, ethanol consumption increased periodontal inflammation, gingival oxidative damage, and the production of TNF- α in periodontal ligament fibroblasts in rats. Ethanol feeding also had an additive effect on polymorphonuclear leukocyte infiltration and gingival oxidative damage, thus increasing the severity of periodontal inflammation in this ligature model of periodontitis.

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